PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 January 1999 (12.01.99)	
International application No. PCT/AU98/00396	**Applicant's or agent's file reference** 2050719/EJH
International filing date (day/month/year) 28 May 1998 (28.05.98)	**Priority date** (day/month/year) 28 May 1997 (28.05.97)
Applicant FOOTE, Simon et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   07 December 1998 (07.12.98)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland** Facsimile No.: (41-22) 740.14.35	Authorized officer S. Cruz Telephone No.: (41-22) 338.83.38

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PATENT COOPERATION TREATY

REC'D 24 AUG 1999

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 2051719/EJH/AF	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International application No. **PCT/AU 98/00396**	International filing date *(day/month/year)* 28 May 1998	Priority Date *(day/month/year)* 28 May 1997
International Patent Classification (IPC) or national classification and IPC **Int. Cl.**[6] G01N 33/483, C12Q 1/527, 1/68		
Applicant THE WALTER & ELIZA HALL INSTITUTE OF MEDICAL RESEARCH		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheet(s).

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☒	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

Date of submission of the demand 7 December 1998	Date of completion of the report 18 August 1999
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. (02) 6285 3929	Authorized Officer **ROSS OSBORNE** Telephone No. (02) 6283 2404

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/AU 98/00396

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed.

[ ] the description, pages , as originally filed,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the claims, pages , as originally filed,
pages , as amended (together with any statement) under Article 19,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the drawings, pages , as originally filed,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , filed with the letter of .

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, was on the basis of the sequence listing:

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages
[ ] the claims, Nos.
[ ] the drawings, sheets/fig.

5. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	6-9, 15-18,20-21, 23, 29-32	YES
	Claims	1-5, 10-14, 19, 22, 24-28	NO
Inventive step (IS)	Claims		YES
	Claims	1-32	NO
Industrial applicability (IA)	Claims	1-32	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

NOVELTY (N): Claims 1-5, 10-14, 19, 22, 24-28

(a) WO 96/29431

The current application teaches a method of detecting a difference in one or more nucleotides (not necessarily of DNA) involving the steps of:

(i) base specific cleavage

(ii) MALDI-TOF-MS

(iii) use of reference nucleic acid molecule to identify peak with alteration of one or more nucleotides

It appears that the use of MALDI-TOF-MS to detect single nucleotide changes in nucleotides that have been digested by endonuclease enzymes only became known in the literature after the earliest priority date of this application (see Y. Wada et al). However, the use of MALDI-TOF-MS to detect changes of more than one nucleotide in enzymatic digests of nucleotides was taught by WO 96/29431. See page 26-27, which discloses the use of digests to differentiate Apolipoprotein isoforms. PCR is also taught by this citation. This clearly discloses the method of claims 1 and 24 and the apparatus of claim 19. It is not clear whether a computer program capable of controlling the method is disclosed as it is not clear what a program must feature to be "capable of controlling" the method, and whether the claims require each step of the method to be controlled by the program. The features of the remaining claims are not taught by any single citation.

INVENTIVE STEP (IS):Claims 1-32

Claims 1-32 lack inventive step over WO 96/29431 when combined with Hahner et al who disclose the use of MALDI with base specific endonuclease digests of RNA (and suggest the extension of the method to DNA) and Talbo et al which discloses the benefits of post source decay for structural analysis of oligonucleotides by MALDI Mass Spectrometry. It would be obvious to vary the method of WO 96/29431 to incorporate the teachings of these citations to achieve MALDI analysis of oligonucleotides using base specific digests and post source decay.

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

Application No. Patent No.	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date (valid claim) *(day/month/year)*
1) P,X, WO 97/33000 A	12 September 1997	4 March 1997	4 March 1996
2) P,X, WO 98/12355 A	26 March 1998	19 September 1997	19 September 1996
3) P,X WO 98/20166 A	14 May 1998	6 November 1997	6 November 1996

1) The features of claims 1-5, 10-14, 19, 22, 24-28 are disclosed. See figures, particularly figure 2.

2) The features of claims 10-14 are disclosed on pages 36-38.

3) The features of claims 1-5 10-14, 19, 22, 24-28 are disclosed on pages 83-84, 168, 173-190.

2. Non-written disclosures (Rule 70.9)

Kind of non-written disclosure	Date of non-written disclosure *(day/month/year)*	Date of written disclosure referring to non-written disclosure *(day/month/year)*

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claim 10 is directed at a computer program per se, although the program is capable of controlling a method.

Claims 12, 14-18 in referring to a method are therefore unclear to the extent they are appended to the "method" of claim 10.

Claims 10-18 are not supported by the description which does not fully disclose the computer program used.

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of : V

New Citations

(b) Y. Wada et al, Rapid Communication in Mass Spectrometry Volume 11, page 1657-1660 (1997) " Detection of Single-nucleotide Mutations Including Substitutions and Deletions by matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry".

(c) S. Hahner et al, Nucleic Acid Research (1997) Volume 25, No. 10, 1957-1964 "Matrix-Assisted laser Desorption/Ionization Mass Spectrometry (MALDI) of endonuclease digests of RNA".

(d) G. Talbo et al, Rapid Communications in Mass Spectrometry Volume 10, page 100-103 (1996) "Aspects of he Sequencing of Carbohydrates and Oligonucleotides by Matrix-Assisted Laser Desorption/Ionization Post-source Decay".

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 2051719/EJH/AF	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International application No. **PCT/AU 98/00396**	International filing date *(day/month/year)* 28 May 1998	Priority Date *(day/month/year)* 28 May 1997

International Patent Classification (IPC) or national classification and IPC

Int. Cl.⁶ G01N 33/483, C12Q 1/527, 1/68

Applicant
THE WALTER & ELIZA HALL INSTITUTE OF MEDICAL RESEARCH

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Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. (02) 6285 3929	Authorized Officer **ROSS OSBORNE** Telephone No. (02) 6283 2404

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/AU 98/00396

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pages , filed with the demand,
pages , filed with the letter of .

[ ] the claims, pages , as originally filed,
pages , as amended (together with any statement) under Article 19,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the drawings, pages , as originally filed,
pages , filed with the demand,
pages , filed with the letter of .

[ ] the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , filed with the letter of .

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
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[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

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1. Statement

Novelty (N)	Claims	6-9, 15-18,20-21, 23, 29-32	YES
	Claims	1-5, 10-14, 19, 22, 24-28	NO
Inventive step (IS)	Claims		YES
	Claims	1-32	NO
Industrial applicability (IA)	Claims	1-32	YES
	Claims		NO

2. Citations and explanations (Rule 70.7)

NOVELTY (N): Claims 1-5, 10-14, 19, 22, 24-28

(a) WO 96/29431

The current application teaches a method of detecting a difference in one or more nucleotides (not necessarily of DNA) involving the steps of:

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INVENTIVE STEP (IS):Claims 1-32

Claims 1-32 lack inventive step over WO 96/29431 when combined with Hahner et al who disclose the use of MALDI with base specific endonuclease digests of RNA (and suggest the extension of the method to DNA) and Talbo et al which discloses the benefits of post source decay for structural analysis of oligonucleotides by MALDI Mass Spectrometry. It would be obvious to vary the method of WO 96/29431 to incorporate the teachings of these citations to achieve MALDI analysis of oligonucleotides using base specific digests and post source decay.

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

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1) The features of claims 1-5, 10-14, 19, 22, 24-28 are disclosed. See figures, particularly figure 2.

2) The features of claims 10-14 are disclosed on pages 36-38.

3) The features of claims 1-5 10-14, 19, 22, 24-28 are disclosed on pages 83-84, 168, 173-190.

2. Non-written disclosures (Rule 70.9)

Kind of non-written disclosure	Date of non-written disclosure (day/month/year)	Date of written disclosure referring to non-written disclosure (day/month/year)

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claim 10 is directed at a computer program per se, although the program is capable of controlling a method.

Claims 12, 14-18 in referring to a method are therefore unclear to the extent they are appended to the "method" of claim 10.

Claims 10-18 are not supported by the description which does not fully disclose the computer program used.

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of : V

New Citations

(b) Y. Wada et al, Rapid Communication in Mass Spectrometry Volume 11, page 1657-1660 (1997) " Detection of Single-nucleotide Mutations Including Substitutions and Deletions by matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry".

(c) S. Hahner et al, Nucleic Acid Research (1997) Volume 25, No. 10, 1957-1964 "Matrix-Assisted laser Desorption/Ionization Mass Spectrometry (MALDI) of endonuclease digests of RNA".

(d) G. Talbo et al, Rapid Communications in Mass Spectrometry Volume 10, page 100-103 (1996) "Aspects of he Sequencing of Carbohydrates and Oligonucleotides by Matrix-Assisted Laser Desorption/Ionization Post-source Decay".

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 2050719/EJH	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. **PCT/AU 98/00396**	International filing date *(day/month/year)* 28 May 1998	(Earliest) Priority Date *(day/month/year)* 28 May 1997

Applicant
(1) THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH
(2) FOOTE, Simon

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I)

2. [ ] **Unity of invention is lacking** (See Box II)

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed
   - [ ] transcribed by this Authority

4. With regard to the **title**,
   - [ ] the text is approved as submitted by the applicant.
   - [X] the text has been established by this Authority to read as follows:

NUCLEIC ACID DIAGNOSTICS BASED ON MASS SPECTROMETRY OR MASS SEPARATION AND BASE SPECIFIC CLEAVAGE

5. With regard to the **abstract**,
   - [ ] the text is approved as submitted by the applicant
   - [X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No.
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure
   - [ ] because this figure better characterises the invention
   - [X] None of the figures

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00396

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

A method of detecting a mutation or a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-ATOF MS and/or other equivalent procedure to produce a fingerprint of then oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00396

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: G01N 33/483, C12Q 1/527, 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC : G01N C12Q and keywords mass spectrom : and oligonucleotide or DNA or nucleic acid

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT (WPAT) and keywords as above
CHEMICAL ABSTRACTS (CA) and keywords as above

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/29431 A (SEQUENOM, INC) 26 September 1996 page 5 lines 13-21, page 6 lines 8-13, figs 7B, 9, 21A-C and claims 33 and 48	1-32
X	WO 96/36986 A (PERSEPTIVE BIOSYSTEMS, INC) 21 November 1996 whole document	1-32
X	WO 97/33000 A (GENETRACE SYSTEMS) 12 September 1997 page 10 line 1 to page 13 line 22, examples, 5, 8 and 11	1-32

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 August 1998	2 SEP 1998

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	A. Achilleos **ANDREW ACHILLEOS** Telephone No.: (02) 6283 2280

International Application No.
PCT/AU 98/00396

C (Continuation)	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98/12355, A (GENETRACE SYSTEMS) 26 March 1998 page 4 line 13 to page 6 line 32, page 9 lines 21 to page 10 line 7, examples 5 to 7	1-32
P,X	WO 98/20166 A (SEQUENOM, INC) 14 May 1998 abstract and claims	1-32

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 98/00396

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	96/29431	AU	53651/96	CA	2214359	EP	815261
		US	6505798				
WO	96/36986	EP	827628				
WO	97/33000	AU	20695/97				
WO	98/12355	AU	45916/97				
WO	98/20166	AU	51069/98				

END OF ANNEX

PCT
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] :
G01N 33/483, C12Q 1/527, 1/68

A1

(11) International Publication Number: WO 98/54571

(43) International Publication Date: 3 December 1998 (03.12.98)

(21) International Application Number: PCT/AU98/00396

(22) International Filing Date: 28 May 1998 (28.05.98)

(30) Priority Data:

PO 7102	28 May 1997 (28.05.97)	AU
PO 7109	30 May 1997 (30.05.97)	AU
PP 1665	5 February 1998 (05.02.98)	AU
PP 3592	19 May 1998 (19.05.98)	AU

(71) Applicant (*for all designated States except US*): THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH [AU/AU]; Royal Parade, Parkville, VIC 3052 (AU).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): FOOTE, Simon [AU/AU]; 19 Park Street, Fairfield, VIC 3078 (AU). ELSO, Colleen [AU/AU]; 26 Christmas Street, Northcote, VIC 3070 (AU). SIMPSON, Richard [AU/AU]; 42 Stanley Street, Richmond, VIC 3121 (AU). REID, Gavin [AU/AU]; 330 Pascoe Vale Road, Essendon, VIC 3040 (AU).

(74) Agents: HUGHES, E., John, L. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: NUCLEIC ACID DIAGNOSTICS BASED ON MASS SPECTROMETRY OR MASS SEPARATION AND BASE SPECIFIC CLEAVAGE

(57) Abstract

A method of detecting a mutation or a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI–ATOF MS and/or other equivalent procedure to produce a fingerprint of then oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

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NUCLEIC ACID DIAGNOSTICS BASED ON MASS SPECTROMETRY OR MASS SEPARATION AND BASE SPECIFIC CLEAVAGE

FIELD OF THE INVENTION

The present invention relates generally to a method for detecting a mutation in a nucleic acid molecule. The method of the present invention does not require prior knowledge of a reference or wild-type nucleotide sequence nor does it require a gel electrophoresis step. The method of the present invention is particularly useful in identifying mutations and polymorphisms in genomic DNA and more particularly in the human genome and to determine and/or confirm the nucleotide sequence of target nucleic acid molecules. The method of the present invention may also be automated.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

The increasing sophistication of recombinant DNA technology is greatly facilitating research and development in a range of biotechnological fields. A particularly important area is the generation of nucleotide mutants and the screening for and identification of such mutants. This in turn has implications, for example, in understanding the genetic basis behind certain disease conditions which is becoming of increasing relevance as the human genome is progressively sequenced.

An efficient and accurate method of mutation detection is crucial in implicating disease candidate genes and in the screening programs which follow identification of disease causing mutations. Many human inherited and sporadic disorders are caused by small mutations including base substitutions, additions and deletions. Among these disorders are the Mendelian single gene disorders, sporadic somatic mutations causing cancers and complex genetic traits. Whilst some diseases are caused by a limited and well characterised set of mutations, most genetic diseases are caused by one or more of a large range of mutations occurring anywhere within the gene. It is important, therefore, that a mutation detection protocol be able to scan a region of DNA,

identify any change and describe the resulting nucleotide differences from wild-type. With the increasing use of population molecular genetics and as clinicians begin to use mutation analysis as a clinical tool, there is a need to develop mutation detection protocols which can be automated, are less dependant on user expertise and are more accurate and reliable.

Current mutation detection protocols require either a gel based detection system or sequence specific primers. Gel based detection methods include direct sequencing of amplified DNA fragments and various techniques involving either cleavage of mismatched bases in heteroduplexes or mobility differences of single or partially denatured DNA strands.

Detection of mutations by DNA sequencing can provide good results in relation to accuracy and information about the position and nature of the mutation (Hattori *et al,* 1993), however, although advances have been made in this area, the technique is not fully automated and is labour intensive. Most mutations occur as heterozygotes and there are technical difficulties with the ability of currently available computer software to identify two different nucleotide bases at a mutated residue.

Many mutation detection techniques exploit differential electrophoretic mobilities of DNA fragments with sequence differences. Single strand conformation polymorphism (SSCP) exploits the fact that the secondary structure of a single strand of DNA is sequence based and, therefore, strands with even just one base difference will migrate at a different rate (Orita *et al,* 1989). This technique is again gel based and can lack sensitivity. Furthermore, the method cannot be readily automated and requires a large amount of labour due to the necessary gel step which in most cases must be optimised to the specific sample being analysed. They also do not give any information about the position or nature of the change and do not routinely identify all mutations.

Mutation detection based on the identification of base pair mismatches in heteroduplex DNA strands is another method of identifying point changes. There are a number of techniques available that cleave DNA at mismatched base pairs in heteroduplex DNA. Mismatch cleavage protocols include chemical and enzymatic mismatch cleavage. The techniques are also gel based. The chemical cleavage method uses osmium tetroxide to cleave at the mismatched base (Cotton

et al, 1988) followed by separation of cleaved products on denaturing gels. A major disadvantage of the chemical cleavage protocol is the use of extremely toxic chemicals.

Other methods for detection of known mutations include minisequencing allele specific polymerase chain reaction (PCR), oligonucleotide probe arrays (Lipshutz *et al,* 1995) which requires knowledge of the sequence of wild-type and mutant. Although this technique is suitable for non-gel based detection methods, it is only useful for know mutations. Furthermore, the large number of oligonucleotides required to cover all known mutations in many genes makes this approach prohibitively expensive and labour intensive.

With the development of the matrix assisted laser desorption ionisation - time of flight mass spectrometer (MALDI-TOF MS), the ability to accurately determine the mass of biomolecules of a limited size has been achieved. Although detection of DNA fragments of up to 622 base pairs in length has been reported, large fragments cannot be accurately sized and a mass accuracy of ±3bp is quoted (Liu *et al,* 1995). This level of accuracy is clearly insufficient for the detection and characterisation of base substitutions.

There is a need, therefore, to develop an effective and accurate means of detecting mutations in nucleic acid molecules. Preferably, the mutation detection system would be automatable.

In work leading up to the present invention the inventors developed a mutation detection system which exploits the accuracy of mass determination of MALDI-TOF MS and which is applicable for large DNA fragments. The method of the present invention do not require gel electrophoresis nor is prior knowledge of the nucleotide sequence necessary. The method of the present invention is also capable of being automated.

SUMMARY OF THE INVENTION

Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

One aspect of the present invention contemplates a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Another aspect of the present invention provides a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule by polymerase chain reaction (PCR), subjecting the test amplified nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Yet another aspect of the present invention is directed to a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule by PCR, subjecting the test amplified nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments of from about 2 to about 1000 bases, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Still yet another aspect of the present invention relates to a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule and incorporating uracil residues, subjecting the test amplified nucleic acid molecule to uracil specific cleavage mediated by a uracil-N-glycosylase to generate oligonucleotide fragments of from about 2 to about 1000 bases, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Another aspect of the present invention contemplates a computer programme capable of controlling a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an

altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Yet another aspect of the present invention is directed to an apparatus capable of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said apparatus comprising means of subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Still another aspect of the present invention provides a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure and subjecting said separated fragments to further separation means, such as post source decay (PSD) or other similar technique, to separate fragmentation products to generate a spectrum dependent on nucleotide sequence and then identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphical representation showing mass spectrogram of cleavage products of two oligonucleotides, 1 and 2, which differ at two nucleotides, one produces a fragment with a different nucleotide composition and the other introducing a new cleavage site. The two line thicknesses represent the overlaid tracings of the two different oligonucleotides. 1636.3 represents a thick line peak and 3190.9 represents a thin line peak. 1811.1 is a thin line peak and 1828.2 is a thick line peak. Kratos Kompact MALDI 4v51.2; % int. 100% = 24mV (thin); 81mV (thick).

Figure 2 is a graphical representation showing mass spectrogram of reacted, separated products of normal TUB which represents a homozygote. Mode: linear; Accelerating Voltage: 20,000; Grid Voltage: 92.000%; Guide Wire Voltage 0-100%; Delay 125ON; Laser:1800; Scans Averaged: 128; Pressure: 9.94e-07; Low Mass Gate: 900.0; Negative Ions: ON.

Figure 3 is graphical representation showing mass spectrogram of reacted, separated products of both TUB-M and TUB which represents a heterozygote. Mode: linear; Accelerating Voltage: 20,000; Grid Voltage: 92.000%; Guide Wire Voltage 0-100%; Delay 125ON; Laser:1800; Scans Averaged: 128; Pressure: 1.89e-06; Low Mass Gate 900.0; Negative Ions: ON.

Figure 4 is a representation of the nucleotide sequence of IL-12 untranslated region PCR product used in Example 13. Primers are shown in bold. Expected cleavage products >2bp are underlined. The polymorphism is at position 97 and is indicated by asterisk. The polymorphism is a C to T change which results in a change of the cleavage products at that position from CGA to AGA in the forward strand and CAAGC to CAA in the reverse stand. The presence of C at position 97 results in a TaqI site and this allele is called "+", the other allele is respectively "-".

Figure 5A is a photographic representation of a TaqI restriction digest of IL-12 PCR products from +/- individuals (lanes 1, 4 and 5), a +/+ individual (lane 3) and a -/- individual (lane 2). The 124 bp fragment is cleaved by TaqI (where possible) to produce 97 and 27 bp fragments.

Figure 5B is a graphical representation showing linear MALDI-TOF spectra of cleavage products. The spectra on the left show a mass range of 1000 to 3500 and those on the right are the same spectra but show in detail the mass range from 1000 to 1700. Spectra *i* a and b are from a -/- individual, spectra *ii* a and b are from a +/+ individual and spectra *iii* a and b are from a +/- individual. Observed masses are indicated above peaks. Arrows show the peaks that change between the two alleles.

Figure 6 is a graphical representation of the mass spectrum analysed using post source decay (PSD) on a MALDI-TOF instrument. Spectrum A is a 6mer of sequence CATCCT [SEQ ID NO:16] and spectrum B a 6mer of sequence CACCTT [SEQ ID NO:17]. Both have parent ion mass of 1727.2Da. Observed masses are shown above the peaks. PSD fragments are shown at an intensity magnification of five.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on a base specific cleavage reaction to generate a set of small oligonucleotides bounded by the base cleaved. The nucleic acid molecule may be completely or only partially cleaved or digested. These fragments are then separated based on mass by MALDI-TOF MS. This generates a fingerprint of the nucleic acid fragment comprising a series of peaks where each peak represents the mass of each small cleavage product. As a result of the sensitivity of mass determination, each oligonucleotide of given length but different nucleotide composition produces a different mass. The mass of each peak, therefore, corresponds to the nucleotide composition of the fragment as well as to its length. Consequently, any nucleotide substitution results in either a shifted peak due to the mass difference in the new cleavage fragment or, if the mutation changes the targeted base, a cleavage product containing a different number of bases.

Accordingly, one aspect of the present invention contemplates a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Conveniently, screening is carried out by comparing the cleavage product masses of the reference or wild-type nucleic acid to those of the test sample. Mass changes corresponding to base changes are readily observed.

Accurate mass determination of these small fragments is possible allowing unambiguous assignation of base composition of each oligonucleotide. This knowledge allows deduction of the nature of the mutation and, after specific cleavage at different bases and integration of the

data, the position of the mutation.

The method of the present invention is applicable to any nucleic acid molecule such as but not limited to DNA, genomic DNA, cDNA, plasmid DNA, satalite DNA, mRNA and other RNA molecules as well as DNA:DNA, DNA:RNA and RNA:RNA hybrids. The present invention is particularly applicable to nucleic acid molecules amplified by, for example, polymerase chain reaction (PCR).

According to this aspect of the present invention, there is provided a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule by polymerase chain reaction (PCR), subjecting the test amplified nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

A particularly preferred requirement is that the source of nucleic acid is cleavable to oligonucleotide fragments of from 2 bases to 1000 bases, preferably of from 3 bases to 500 bases, more preferably of from 4 bases to 100 bases and even more preferably of from 4 bases to 50 bases. Oligonucleotide fragments of form 4 bases to 40 bases are of particular usefulness in practising the present invention.

Accordingly, the present invention is directed to a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule by PCR, subjecting the test amplified nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments of from about 2 to about 1000 bases, separating the resulting oligonucleotide

fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

The nucleic acid may be cleaved by a range of chemical molecules including enzymes. Enzymes are particularly preferred due to their specificity. One useful enzyme is uracil-N-glycosylase which cleaves DNA at uracil residues incorporated, for example, during a PCR. However, a range of enzymes may be employed.

According to this embodiment, the present invention relates to a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising amplifying said test nucleic acid molecule and incorporating uracil residues, subjecting the test amplified nucleic acid molecule to uracil specific cleavage mediated by a uracil-N-glycosylase to generate oligonucleotide fragments of from about 2 to about 1000 bases, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

The method of the present invention is predicated in part on the fact that any oligonucleotide fragment differing in nucleotide composition between mutant and wild-type (or reference) sequences will be detected. The method has advantages over previously employed techniques and such advantages include the absence of a gel electrophoresis step thereby reducing time, expertise and need for separation equipment and the lack of dependance on toxic chemicals, such as osmium tetroxide. Whilst the present invention extends to the use of such chemicals in base specific cleavage reactions, it is preferred to use an enzymatic reaction to cleavage the target nucleic acid molecule.

The method of the present invention is particularly useful in detecting previously unknown mutations. This is important as a screening mechanism for inherited diseases and cancers such as during pre-natal diagnosis, diagnosis of a suspected disease or screening for carriers of disease alleles. It also has applications in polymorphism analysis of populations and in studies of evolution, drug resistance, virulence or attenuation of disease agents such as bacteria, viruses or protozoa.

The method may be carried out simultaneously or sequentially with an analysis of a reference to wild-type nucleic acid molecule. Both the test and reference nucleic acid molecules can then be compared. Alternatively, the wild-type nucleic acid molecule may already have been analysed. Conveniently, this information may be stored electronically and upon completion of the analysis of the test nucleic acid molecule, both the test and reference sequences may then be compared manually, electronically or by a computer assisted means.

The method of the present invention may also be used to determine the nucleotide sequence of a nucleic acid molecule.

The nucleotide sequence may be completely determined or a partial sequence obtained for example, for selected nucleotides. The method of the present invention, therefore, permits the rapid determination of a nucleotide sequence which will be invaluable, for example, in the efficient analysis of mutations.

The method of the present invention may be semi or fully automated and the present invention extends to apparatuses for automating the mutation detection assay. The apparatus may also be electronically controlled by a computer programme to facilitate the automation and/or analysis process.

Accordingly, another aspect of the present invention contemplates a computer programme capable of controlling a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide

fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Yet another aspect of the present invention is directed to an apparatus capable of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said apparatus comprising means of subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

In a particularly preferred embodiment, the method of apparatus of the present invention also employs a further fragment separation means such as but not limited to post source decay (PSD). PSD, for example, uses the dissociation of highly energised ions during their flight to the detector creating a second dimension. The ions are directed into an electric field of opposite polarity and are reflected. Smaller ions are reflected earlier and reach the detector first. As the spectrum from the decay is dependent on the nucleotide sequence of an oligonucleotide rather than the nucleotide composition, this avoids missing mutations in an oligonucleotide having the same nucleotide composition as a reference oligonucleotide. Although PSD is one convenient fragment separation means, the present invention extends to other similar techniques to separate fragmentation products. Generally these techniques are based on mass although may also be based on electrophoretic mobility, base size, base charge, base paring or other suitable criteria.

Accordingly, another aspect of the present invention provides a method of detecting a difference

of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure and subjecting said separated fragments to further separation means to generate a spectrum dependent on nucleotide sequence and then identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

The MALDI-TOF MS analysis and further separation means may be done sequentially or simultaneously.

Preferably, the further separation means includes or comprises PSD or other similar techniques to separate fragmentation products.

The present invention is particularly useful in identifying and/or locating mutants in heterozygotes. Mutations are detectable on both strains or on one strand only.

Yet another aspect of the present invention provides a method for identifying and/or locating a mutation in one or more bases in a target nucleic acid molecule, subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

Preferably, the separated fragments are subjected to further separation means such as but not limited to PSD.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

OLIGONUCLEOTIDES

Two test 22mers oligonucleotides with two bases different were used in this study

CCT C**A**T UTT TTU TTG T**A**A GAG G [SEQ ID NO:1]

CCT C**G**T UTT TTU TTG T**U**A GAG G [SEQ ID NO:2]

The different bases are shown in bold.

For the detection of point mutations (see Example 7), the following oligonucleotides are used:

TUB:
GGT GAC CTG AAC CAC CTC GTG CGT CCA GCC GTT CG**T** GGC TGT CCA GTC CGC GAAC TCT GAC CTG CGC AAG [SEQ ID NO:3]

TUB-M:
GGT GAC CTG AAC CAC CTC GTG CGT CCA GCC GTT CG**A** GGC TGT C**G**A GTC CGCGAA CTC TGA CCT GCG CAA G [SEQ ID NO:4]

TUB-F:
GGT GAC CTG AAC CAC CTC GT [SEQ ID NO:5]

TUB-R:
CTT GCG CAG GTC AGA GTT [SEQ ID NO:6]

TUB and TUB-M are used as template DNA and differ at three residues, bolded above, which comprise two point mutations and one insertion (bracketed and bolded). TUB-F and TUB-R are the "reverse" and "forward" primers used to amplify either TUB or TUB-M in a polymerase chain reaction.

EXAMPLE 2
CLEAVAGE REACTION

The cleavage reactions were carried out using 100 pmol of oligonucleotide, 0.5 units uracil -N-glycoslyase (Perkin - Elmer) 1xPCR buffer (50mM KCl, 10mM Tris-HCl pH 8.3) (Perkin-Elmer) in a 250μl reaction. The reaction mixture was incubated at 50°C for 20 minutes to allow cleavage of the N-glycosidic bond at uracil. It was then heated for 15 minutes to 105°C to allow degradation of the phosphate bonds at the basic sites. The mixture was then purified using anion exchange resin to remove buffer salts and other impurities.

EXAMPLE 3
SAMPLE PURIFICATION

Qiagen Anion Exchange Resin was equilibrated in 5mM NH_4HCO_3 (Sigma) pH 8.4 (sodium free). 40μl of the slurry was added to the reaction mixture and the DNA was allowed to bind at room temperature for 5 minutes with gentle shaking. The beads were spun down in a bench centrifuge and the supernatant discarded. The beads were then washed with 3x100μl volumes of 5mM NH_4HCO_3 pH 8.4 (sodium free) with incubation and centrifugation between each wash. The supernatant was discarded each time. The DNA fragments were then eluted using two 40μl volumes of 0.5M NH_4HCO_3 pH 8.0 (sodium free), with incubation and centrifugation as before but with the supernatant being kept. The supernatant was then evaporated to dryness on a Savant Speedivac and resuspended twice in 20μl distilled water and evaporated to dryness to remove any residual NH_4HCO_3. The final product was resuspended in 5μl distilled water. The final concentration being approximately 20pmol/μl.

EXAMPLE 4
THE POLYMERASE CHAIN REACTIONS AND DNA URACIL GLYCOSYLASE REACTION

20 ul reactions were set up containing 2.5mM $MgCl_2$, 2.5 mM dATP, dCTP, dGTP, 5 mM dUTP, 0.5U Taq Gold (Perkin Elmer), 1.5 mM each TUB-F and TUB-R oligonucleotides and

2.4 fg or either TUB or TUB-M or a mix of both. PCR assays were incubated at 95°C for 15 minutes then cycled at 95°C - 15 seconds, 60°C - 35 seconds, 72°C - 35 seconds for 40 cycles. PCR reactions were pooled, each pool contained either 10 or 100 PCR reactions. Uracil DNA glycosylase (Perkin Elmer) was added at a ratio of 1U per 10 PCR reactions. Completeness of digestion was confirmed by agarose gel electrophoresis.

EXAMPLE 5
PURIFICATION OF DIGESTED PCR PRODUCTS

Each DNA glycosylase reaction was loaded onto a C8 aquapore RP300 column equilibrated with 0.1M TEAA, the column washed with 0.1M TEAA at a flow rate of 0.5 ml/min and elute with 0.1M TEAA in 60% v/v CH_3CN. Peaks were collected. Column eluates were desiccated on a Savant Speedivac, evaporative centrifuge, resuspended in water to the original volume and redessicated. Pellets were resuspended in 5 ml H_2O. Mass spectrometric samples were prepared as described in Example 6.

EXAMPLE 6
MASS ANALYSIS

3-Hydroxypicolinic acid is prepared at a concentration of 75mg/ml in 1:1 acetonitrile and water and stored at room temperature in a closed vial in the dark. A new matrix solution is prepared weekly. Cation exchange beads (Bio-Rad, 50W-X4, mesh size 100-200μm) in ammonium form were used to reduce interference from sodium and potassium adducts (Nordhoff *et al*, 1992). Samples were prepared as follows: 0.5μl matrix, 0.5μl sample (10pmol DNA) and 0.5μl cation exchange resin were mixed on the slide and allowed to dry. The beads were then blown off with nitrogen gas. Samples were then analysed immediately.

Samples were run on the Kratos Kompact MALDI 4 with 337nm laser or a Perspective Voyager MALDITOF machine. Linear negative mode was used for all spectra. Fifty shots were fired at power setting 70 to find a sweet spot and then a further 50 shots were fired at the sweet spot to obtain the spectrum.

EXAMPLE 7
SIMULATION

In order to assess the ability of this technique to detect mutations, a computer simulation was designed. Two different stimulations were conducted, one that models a mutation occurring in a haploid genome and the other modelling a mutation occurring in a diploid genome on the background of a wildtype sequence.

In order to optimise the detection of mutations, four separate base specific cleavage reactions have been performed using separated forward and reverse strands and two different base specific reagents, in this case, thymidine and cytosine. A random library of exonic sequences has been extracted from Genbank. This comprises 100,000 kb of coding sequence concatenated into one file. Sequence strings of incremental length are removed from this file. A fingerprint for each strand is generated. This is calculated by generating the sets of post cleavage fragments for each base-specific reagent and sorting the non-redundant fragments. Mutant sequences are created by mutating every residue in the wild-type sequence to each of three possible alternatives. The fingerprint of each mutant is generated and compared to the wild-type fingerprints. If the fingerprints are different, it is recorded as a successful detection and the next mutant examined. If the first base specific cleavage reaction is unable to detect the mutation on the forward strand, the reverse strand is tried and so on until the reverse strand of the second reagent fails. This represents the total failure rate under the described conditions. Conceivably one could increase the power of the technique by using all four base specific reagents on both strands.

EXAMPLE 8
DETECTION OF BASE MUTATIONS

Overlaid tracings from the mass spectrogram are presented in Figure 1. These show the cleavage products of two oligonucleotides 1 and 2 [SEQ ID NO:1 and SEQ ID NO:2, respectively], which differ at two nucleotides, one producing a fragment with a different nucleotide composition and the other introducing a new cleavage site. The new fragments resulting from these differences are easily separated. This example, observed masses deviate from calculated

by ±0.02-1%. This is sufficient to assign the correct base composition in this case, however, it is not sufficient to blindly assign base composition peaks from a sample of unknown sequence. A study has been done which concluded that all base compositions can be uniquely specified up to the 14mer level if one base has a known composition (ie. G=1 in the case of the study, or in our case, T=0) with a measurement of mass to within ±0.01%. This is presently achievable, dependent on the mass analyser used and the sample quality and quantity (Pomerantz *et al*, 1993).

Base specific cleavage and mass spectrometry is, therefore, able to differentiate between two identical length oligonucleotides with different nucleotide compositions and hence is able to differentiate between two sequences differing at one base (Table 1). Where a mutation changes the residue involved directly in the base specific cleavage reaction (a "U" residue in the case presented here), the difference in size of the resultant products is marked (Table 1). The accuracy of mass determination allows deduction of the base composition of each fragment and therefore, where the sequence is known, will enable deduction of the nature of the mutation.

Table 2 presents stimulation date for the haploid genome case and Table 3 presents the stimulation data where a mutation occurs in a diploid organism in the presence of a wild-type copy. These data are presented as cumulative "failure to identify" mutations based on both strands and two base specific cleavage reactions. Therefore, the last column, which is where the "C" reaction was unable to pick the mutation on the complementary strand represents the "total failure rate" of the technique under these conditions.

EXAMPLE 9

DETECTION OF POINT MUTATIONS

The method of the present invention has been employed on PCR products and is able to detect point mutations and an insertion in DNA that has been amplified using the polymerase chain reaction as discussed below. The PCR templates used, TUB and TUB-M are described in Example 1 and have three differences, two of which are point mutations and the third is an insertion/deletion. All of these differences are visible in the mass spectrograms (Figures 2 and

3). Figure 3 represents the reacted, separated products of both TUB-M and TUB. This is a reconstruction of a heterozygote. Figure 2 is reacted, separated products of TUB, representing, in this case a homozygote normal. Table 4 gives the expected masses for each fragment and the corresponding comments on whether they have been seen. All mutations were seen on either both strands or on one strand only.

TABLE 1

oligo1:cleavage products		calc. mass	obs.mass
a	CCTCAT[1]	1810.2	1811.1
b	TTTT	1318.8	1318.4
c	TTGTAAGAGG[2]	3190.0	3190.9

oligo2:cleavage products			
a	CCTCGT[3]	1826.2	1828.2
b	TTTT	1318.8	1318.4
c	TTGT	1343.8	1343.5
d	AGAGG[4]	1635.0	1636.3

1 SEQ ID NO:18

2 SEQ ID NO:19

3 SEQ ID NO:20

4 SEQ ID NO:21

Table 2.

Sequence length	Total number wildtype sequences	Total number mutated sequences	Number of "T" forward failures	% "T" forward failures	Number of "T" reverse failures	% "T" reverse failures	Number of "C" forward failures	% "C" forward failures	Number of "C" reverse failures	% "C" reverse failures
40	2500	300000	10569	3.52	97	0.03	1	0		0
60	1666	299880	14723	4.91	237	0.08	14	0	1	0
80	1250	300000	18908	6.30	401	0.13	26	0.01		0
100	1000	300000	22825	7.61	653	0.22	43	0.01		0
120	833	299880	26383	8.80	931	0.31	72	0.02	2	0
140	714	299880	29751	9.92	1225	0.41	93	0.03	8	0
160	625	300000	32644	10.88	1480	0.49	145	0.05	11	0
180	555	299700	35692	11.91	1955	0.65	188	0.06	21	0.01
200	500	300000	38848	12.95	2356	0.79	264	0.09	24	0.01
220	454	299640	40970	13.67	2828	0.94	348	0.12	32	0.01
240	416	299520	43995	14.69	3449	1.15	500	0.17	62	0.02
260	384	299520	46387	15.49	3923	1.31	541	0.18	88	0.03
280	357	299880	48443	16.15	4386	1.46	643	0.21	50	0.02
300	333	299700	50812	16.95	4904	1.64	759	0.25	103	0.03
320	312	299520	52651	17.58	5700	1.90	949	0.32	142	0.05
340	294	299880	54768	18.26	6130	2.04	1082	0.36	155	0.05
360	277	299160	56876	19.01	6621	2.21	1221	0.41	188	0.06
380	263	299820	59231	19.76	7445	2.48	1400	0.47	236	0.08
400	250	300000	60891	20.30	7906	2.64	1507	0.50	240	0.08
1000	100	300000	98902	32.97	27716	9.24	10255	3.42	3798	1.27

Table 3.

Sequence Length	Total number wildtype sequences	Total num,ber mutated sequences	Number of "T" forward failures	% "T" forward failures	Number of "T" reverse failures	% "T" reverse failures	Number of "C" forward failures	% "C" forward failures	Number of "C" reverse failures	% "C" reverse failures
40	2500	300000	120699	40	39139	13	11468	4	3993	1
60	1666	299880	132516	44	48716	16	16455	5	6426	2
80	1250	300000	142523	48	57790	19	22043	7	9600	3
100	1000	300000	150441	50	65780	22	27368	9	12556	4
120	833	299880	156926	52	73381	24	32832	11	16347	5
140	714	299880	163973	55	81099	27	38470	13	20145	7
160	625	300000	169582	57	87388	29	43686	15	23767	8
180	555	299700	173319	58	92501	31	48016	16	27141	9
200	500	300000	178247	59	98957	33	53122	18	30973	10
220	454	299640	181728	61	103638	35	57622	19	34684	12
240	416	299520	184465	62	107959	36	62015	21	38330	13
260	384	299520	188025	63	112626	38	65898	22	41402	14
280	357	299880	191722	64	117075	39	70162	23	45504	15
300	333	299700	194210	65	120601	40	73900	25	48553	16
320	312	299520	196727	66	124257	41	77929	26	51963	17
340	294	299880	199290	66	127979	43	81308	27	54874	18
360	277	299160	200599	67	131116	44	84764	28	58213	19
380	263	299820	203752	68	134422	45	87956	29	60909	20
400	250	300000	205749	69	137686	46	91319	30	64056	21
420	238	299880	207767	69	140874	47	94747	32	67463	22
440	227	299640	209037	70	143182	48	96836	32	69713	23
460	217	299460	210530	70	145413	49	99414	33	71853	24
480	208	299520	212472	71	147958	49	102503	34	74722	25
500	200	300000	214285	71	149928	50	104741	35	77298	26
520	192	299520	215159	72	152227	51	107376	36	79507	27
540	185	299700	215902	72	153784	51	109511	37	81602	27

TABLE 4

EXPECTED TUB FRAGMENTS		FRAGMENTS NOT SEEN	
GGC	1045.6		
CCAC	1198.8		
CCACA [SEQ ID NO:22]	1512*		
CCAG	1318.8		
GGAC	1358.8		
CCAGCCG [SEQ ID NO:23]	2226.4		
GCGCAAG [SEQ ID NO:24]	2210.4		
GCGCAAGA [SEQ ID NO:25]	2523.6*		
CCGCGAAC [SEQ ID NO:26]	2539.6		
GGAGCACGCAGG [SEQ ID NO:7]	3880.4		
CGGCAAGCACCGACAGG [SEQ ID NO:8]	5374.4		
GGTGACCTGAACCACCTCGTGCG [SEQ ID NO:9]	5888.8	PRIMER	
CAGGCGCTTGAGACTGGACGCGT [SEQ ID NO:10]	6258	PRIMER	
EXPECTED TUB-M FRAGMENTS			
CCAC	1198.8	END	
CGAG	1358.8		
GGAC	1358.8		
CGAGGC [SEQ ID NO:27]	1977.2		
CCAGCCG [SEQ ID NO:28]	2226.4		
GCGCAAG [SEQ ID NO:29]	2210.4		
CGACAGCC [SEQ ID NO:30]	2539.6		
CCGCGAAC [SEQ ID NO:31]	2539.6		
CGAACGGC [SEQ ID NO:32]	2579.6		
GGAGCACGCAGG [SEQ ID NO:11]	3880.4		
GGTGACCTGAACCACCTCGTGCG [SEQ ID NO:12]	5888.8	PRIMER	
CAGGCGCTTGAGACTGGACGCGT [SEQ ID NO:13]	6258	PRIMER	

* Fragments obtained due to the terminal transferase activity of Taq polymerase which results in the addition of a dATP at the 3′ end of the PCR product.

EXAMPLE 10

MODIFICATION DETECTION PROTOCOL

The method of Example 8 is employed except DNA polymerase enzymes are employed with the ability to incorporate both dNTPs and rNTPs. Specific cleavage reactions are performed on PCR products in which one of the nucleotides is substituted for rNTP. This permits the base specific cleavage reactions to be conducted in alkali at high temperature.

EXAMPLE 11

IDENTIFICATION OF MUTATION POSITION

The method of Example 8 employs Uracil-N-glycososylase which cleaves DNA at uracil. It is, therefore, a T reaction as uracil is replacing thymidine in the PCR product. In this Example, cleavage occurs at each of other bases so as to create sets of overlapping data to give information about the position of the mutation.

EXAMPLE 12

DETERMINATION OF NUCLEOTIDE SEQUENCE

The method of the present invention is used to determine a nucleotide sequence of a nucleic acid fragment. The method employed is substantially as described in Example 8.

EXAMPLE 13

DETECTION OF PREVIOUSLY UNKNOWN MUTATIONS

The method of the present invention is further demonstrated on a sequence polymorphism in the IL-12 gene. This previously unreported sequence change results in a TaqI RFLP and, therefore, can be followed by enzymatic digestion of PCR products.

Methods

Template DNA was genomic DNA from human volunteers of each possible genotype of the IL-12 polymorphism (ie. +/+, +/-, and -/-, where + is the presence of the Taq restriction site). PCRs were carried out in 20μl reactions in 192 well plates in a Corbett Thermocycler with the following reaction mixture: 50mM KCl, 10mM Tris-HCl pH 8.3, 25mM $MgCl_2$ 2.5mM dATP, dCTP and dGTP (Promega), 5mM dUTP (Boehringer Mannheim GmbH), 0.5U AmpliTaq Gold (Perkin Elmer), 0.4μM primers (Bresatec). After an initial 15min incubation at 95°C, the reactions were cycled 95°C 15 secs, 58°C 35sec, 72°C 35sec, for 40 cycles. 7 reactions were pooled for the homozygotes and 9 for the heterozygote. 1 unit of AmpErase Uracil-N-glycosylase (Perkin Elmer) was added to each pool and the reaction incubated at 50°C for 1 hour, followed by 30 minutes at 105°C. The extend of completion of the cleavage reaction was monitored by the absence of a band on an agarose gel. The cleavage reaction was monitored by the absence of a band on an agarose gel. The cleavage products were purified using reverse phase HPLC on a 100x2.1mm C8 aquapore RP300 column (Applied Biosystems). The flow rate was 0.5ml/min and absorbance was monitored at 254nm. The sample washed with 0.1M triethylaminoacetate (TEAA) and eluted in 0.1M TEAA/60% w/v acetonitrile and the fraction with absorbance at 254nm was collected and evaporated to dryness using a Savant Speedivac. The residue was resuspended in 100μl distilled deionised water and evaporated to dryness and then resuspended in 1μl water. 0.5μl of this was mixed with 0.5μl 3-hydroxypicolinic acid (saturated solution in 50% w/v acetonitrile and 0.5μl NH_4^+ ion-exchange beads (BioRad, 50W-X4, mesh size 100-200μm) on a sample slide. The mass spectrometer used to characterise the reaction products was a Voyager BioSpectrometry Workstation from PerSeptive Biosystems. 128 laser pulses at power 1800 were averaged. Post Source Decay spectra were collected using a Kratos Kompact MALDI4 TOF mass spectrometer with 377nm laser and a curved field reflector in positive ion mode. Matrix and sample preparation as above. After scanning in linear mode for the sweet spot, the ion gate was set 34.8 Da above and 36.2 Da below the parent ion at 1727.2 Da. 200 profiles at 5 shots per profile were averaged. Spectra were corrected for the curved field.

Genotypes were confirmed by demonstrating the presence or absence of the TaqI restriction site

by digesting PCR products with TaqI restriction enzyme (Gibco-BRL) and analysing the products by agarose electrophoresis. DNA bands were stained with ethidium bromide.

A computer simulation of the method has been written and 100kb of random coding sequence from Genbank has been fed into it. The program takes discrete-length bites of sequence from a file of concatenated cDNA sequence from Genbank. Each base is mutated to each hypothetical variant of the original sequence by removing the cleaved base leaving the residual short strings. The mass spectrometry was modelled, fragments of different nucleotide composition being distinguishable and those of identical composition being indistinguishable. As quantitation is difficult on the MALDI, changes in peak height was not used as an indication of a change in underlying sequence. The program then compares "spectra" and tallies the number of mutations that were missed. The program can model the detection of a mutation in the presence of a wild-type sequence (heterozygote) or can model the differences between two homozygotes. In the first case a mutation can only be detected by the presence of a new peak and in the latter case, as well as the presence of a new peak, the disappearance of a peak can also signal a change. All four base specific cleavage reactions were used and reactions were performed on separated strands giving a total of 8 reactions per PCR product. Also the model has been refined to take account of the ability of post source decay (PSD) to identify changes in peaks containing a complex mix of oligonucleotides. In this case fragments of different sequence are distinguishable.

Results

A PCR assay was designed to incorporate the mutated region and then subjected to uracil -N glycosylase treatment. The products were purified and analysed by MALDI-TOF mass spectrometry. The sequence of the PCR primers and product along with the mutation are shown in Figure 4. The C to T change gives rise to a Taq RFLP and this can be seen in homozygote and heterozygote state in Figure 5. The spectra generated by the MALDI-TOF can also be seen in Figure 5. The expected and observed masses of the cleavage products from the two alleles are given in Table 5. The position of the mutation and deduction of the changed base is evident from study of this Table.

A limitation to the sensitivity of this method results from the lack of quantitative data available from the MALDI. When the fragment derived from the mutated sequence coincides with other fragments of identical nucleotide composition in the wild-type sequence, its disappearance will go undetected. Similarly, the appearance of a new fragment in the mutated sequence will go unnoticed of it has identical nucleotide composition to one or more other cleavage products. If both these conditions exist for all cleavage reactions, then the mutation will be missed. This technique, therefore, is not as advantageous for longer fragment as for small fragments.

To address this problem, the inventors employed a second dimension detection protocol on the MALDI-TOF machine. Post source decay (PSD) uses the dissociation of the highly energised ions during their flight to the detector as this second dimension. They are directed into an electric field of opposite polarity and are reflected. The smaller ions are reflected earlier and reach the detector first. As the spectrum from the decay is dependent on the sequence of the oligonucleotide (and not the nucleotide composition), the aforementioned limitation is bypassed, generating a method of mutation detection that is now extremely sensitive.

The utility of MALDI-TOF analysis with PSD is demonstrated in Figure 6 where two oligonucleotides of identical nucleotide composition are separated by MALDI-TOF using PSD. The resulting spectra are quite distinguishable. Sequence determination of small oligonucleotides is feasible using molecular dissociation methods and, therefore, the subject method extrapolates into an accurate resequencing protocol.

A computer simulation of data from the linear separation of cleavage products has been written. Using Genbank data, the expected number of base substitution that would be identified when comparing two homozygotes over a 250bp PCR distance is 98.5%. the comparable figure is 95% when a homozygote is compared to a heterozygote. If each mass peak from a base specific cleavage is analysed using a secondary dissociation technique, eg. PSD on the MALDI-TOF machine, then sensitivity of mutation detection improves dramatically. This has also been simulated and for a 1000bp fragment subjected to base specific cleavage, and analysed with PSD, 99% of all substitutions will be detected for a homozygote to heterozygote comparison and 99.8% when two homozygotes are compared.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(US only): FOOTE Simon, ELSO Colleen, SIMPSON Richard, REID Gavin

(ii) TITLE OF INVENTION: A NOVEL METHOD

(iii) NUMBER OF SEQUENCES: 32

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT INTERNATIONAL
(B) FILING DATE: 28 MAY 1998
(C) CLASSIFICATION:

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(B) FILING DATE: 19-MAY-1998

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTC**A**TUTTT TUTTGT**A**AGA GG 22

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTC**G**TUTTT TUTTGT**U**AGA GG 22

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGTGACCTGA ACCACCTCGT GCGTCCAGCC GTTCG**T**GGCT GTCCAGTCCG 50
CAAACTCTGA CCTGCGCAAG 70

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGTGACCTGA ACCACCTCGTG CGTCCAGCCG TTCG**A**GGCTG TC**G**AGTCCGC 50
(**G**)AACTCTGAC CTGCGCAAG 69

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTGACCTGA ACCACCTCGT 20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTTGCGCAGG TCAGAGTT 18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGAGCACGCAG G 11

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGCAAGCAC CGACAGG 17

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGTGACCTGA ACCACCTCGT GCG 23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGGCGCTTG AGACTGGACG CGT 23

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGAGCACGCA GG 12

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTGACCTGA ACCACCTCGT GCG 23

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAGGCGCTTG AGACTGGACG CGT 23

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 124 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACAACGGAA TAGACCCAAA AAGAUAAUUU CUAUCUGAUU UGCUUUAAAA 50
CGUUUUUUUA GGAUCACAAU GAUAUCUUUG CUGUAUUUGU AUAGUUCGAU 50
GCUAAAUGCU CAUUGAAACA AUCA 24

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 124 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GUGUUGCCUU AUCUGGGUUU UUCUAUUAAA GAUAGACUAA ACGAAAUUUU 50
GCAAAAAAAU CCUAGUGUUA CUAUAGAAAC GACAUAAACA UAUCAAGCUA 50
CGATTTACGA GTAACTTTGT TAGT 24

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CATCCT 6

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CACCTT 6

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCTCAT 6

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TTGTAAGAGG 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCTCGT 6

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGAGG 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCACA 5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCAGCCG 7

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCGCAAG 7

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GCGCAAGA 8

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CCGCGAAC 8

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CGAGGC 6

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CCAGCCG 7

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

GCGCAAG 7

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

CGACAGCC 8

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CCGCGAAC 8

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

CGAACGGC 8

CLAIMS:

1. A method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

2. A method according to claim 1 wherein the nucleic acid molecule to be tested is amplified by a polymerase chain reaction (PCR) prior to base specific cleavage.

3. A method according to claim 1 or 2 wherein the base specific cleavage results in oligonucleotide fragments of from about 2 bases to about 1000 bases.

4. A method according to claim 3 wherein the base specific cleavage results in oligonucleotide fragments of from about 3 bases to about 500 bases.

5. A method according to claim 4 wherein the base specific cleavage results in oligonucleotide fragments of from about 4 bases to about 100 bases.

6. A method according to any one of claims 1 to 5 wherein the base specific cleavage is uracil specific cleavage.

7. A method according to claim 6 wherein the uracil specific cleavage is mediated by uracil-N-glycosylase.

8. A method according to any one of claims 1 to 7 further comprising subjecting

fragmentation products to further separation (PSD) to generate a spectrum from decay dependent on the nucleotide sequence of the oligonucleotide.

9. A method according to claim 8 wherein the further separation of fragmentation products is by post source decay (PSD).

10. A computer programme capable of controlling a method of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said method comprising subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

11. A method according to claim 9 wherein the nucleic acid to be tested is amplified by PCR prior to base specific cleavage.

12. A method according to claim 9 or 10 wherein the base specific cleavage results in oligonucleotide fragments of from about 2 bases to about 1000 bases.

13. A method according to claim 9 wherein the base specific cleavage results in oligonucleotide fragments of from about 3 bases to about 500 bases.

14. A method according to claim 10 wherein the base specific cleavage results in oligonucleotide fragments of from about 4 bases to about 100 bases.

15. A method according to any one of claims 9 to 13 wherein the base specific cleavage is uracil specific cleavage.

16. A method according to claim 14 wherein the uracil specific cleavage is mediated by uracil-N-glycosylase.

17. A method according to any one of claims 10 to 16 further comprising the further separation of fragmentation products to generate a spectrum from decay dependent on the nucleotide sequence of the oligonucleotide.

18. A method according to claim 17 wherein the further separation of fragmentation products is by post source decay (PSD).

19. An apparatus capable of detecting a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule, said apparatus comprising means of subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

20. An apparatus according to claim 19 further comprising further fragmentation separation means to generate a spectrum from decay dependent on the nucleotide sequence of the oligonucleotide.

21. An apparatus according to claim 20 wherein the further fragmentation separation means is post source decay (PSD).

22. Use of MALDI-TOF in the detection of a difference of one or more nucleotides between a nucleic acid molecule to be tested and a reference nucleic acid molecule.

23. Use according to claim 22 further comprising use of PSD to generate a spectrum for

decay dependent on the sequence of an oligonucleotide.

24. A method for identifying and/or locating a mutation in one or more bases in a target nucleic acid molecule, subjecting the test nucleic acid molecule to base specific cleavage to generate oligonucleotide fragments, separating the resulting oligonucleotide fragments based on mass by MALDI-TOF MS and/or other equivalent procedure to produce a fingerprint of the oligonucleotide fragments comprising one or more peaks wherein a peak represents the mass of each fragment and identifying an altered peak relative to a reference nucleic acid molecule subjected to the same procedure wherein the presence of an altered peak is indicative of a difference of one or more nucleotides in said tested nucleic acid molecule.

25. A method according to claim 24 wherein the nucleic acid molecule to be tested is amplified by a polymerase chain reaction (PCR) prior to base specific cleavage.

26. A method according to claim 24 or 25 wherein the base specific cleavage results in oligonucleotide fragments of from about 2 bases to about 1000 bases.

27. A method according to claim 26 wherein the base specific cleavage results in oligonucleotide fragments of from about 3 bases to about 500 bases.

28. A method according to claim 27 wherein the base specific cleavage results in oligonucleotide fragments of from about 4 bases to about 100 bases.

29. A method according to any one of claims 24 to 28 wherein the base specific cleavage is uracil specific cleavage.

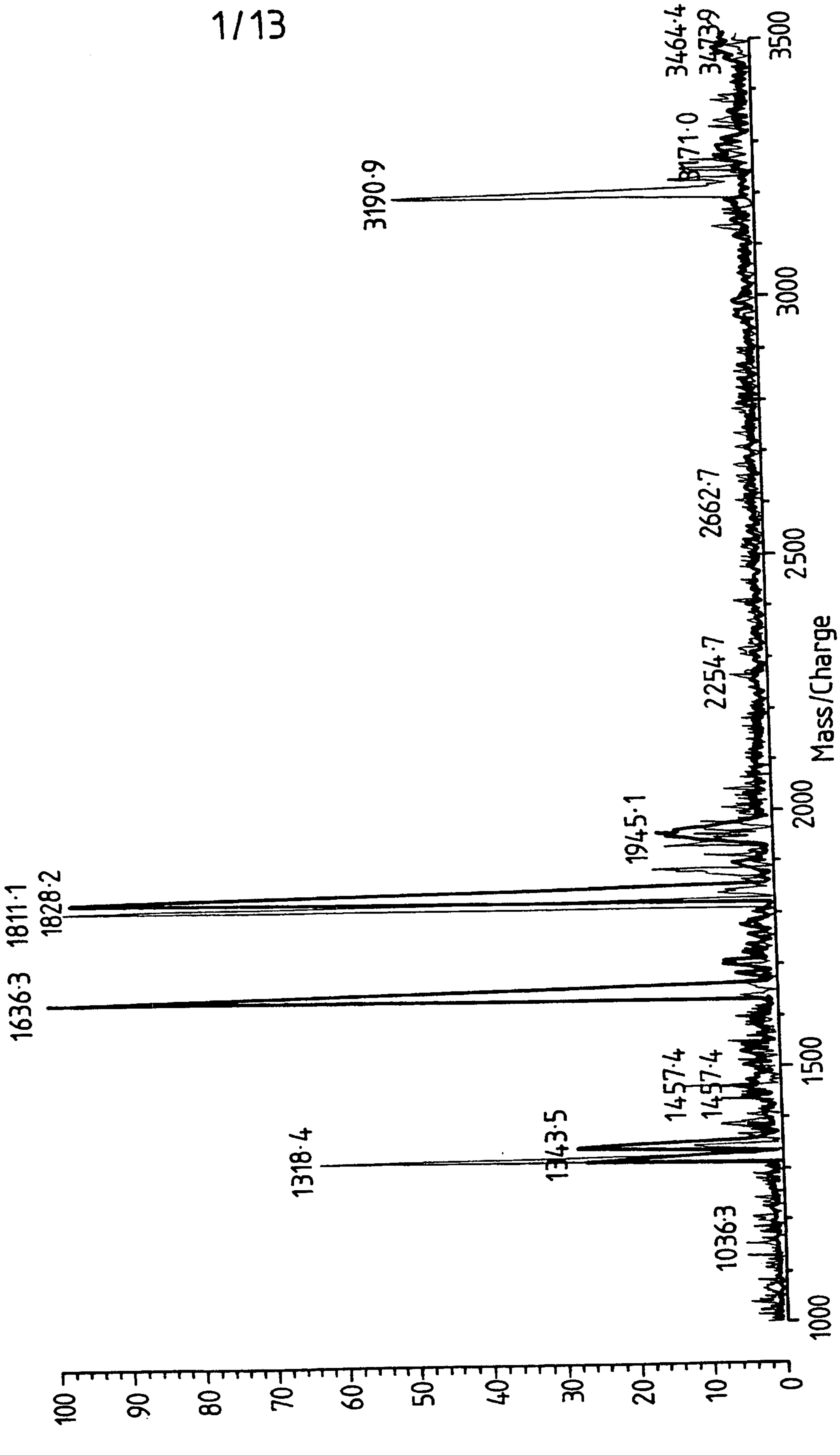
30. A method according to claim 29 wherein the uracil specific cleavage is mediated by uracil-N-glycosylase.

31. A method according to any one of claims 24 to 30 further comprising subjecting fragmentation products to further separation (PSD) to generate a spectrum from decay

dependent on the nucleotide sequence of the oligonucleotide.

32. A method according to claim 31 wherein the further separation of fragmentation products is by post source decay (PSD).

FIG 1



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3171·0
3190·9
2662·7
2254·7
1945·1
1811·1
1828·2
1636·3
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1457·4
1343·5
1318·4
1036·3
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60
70
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90
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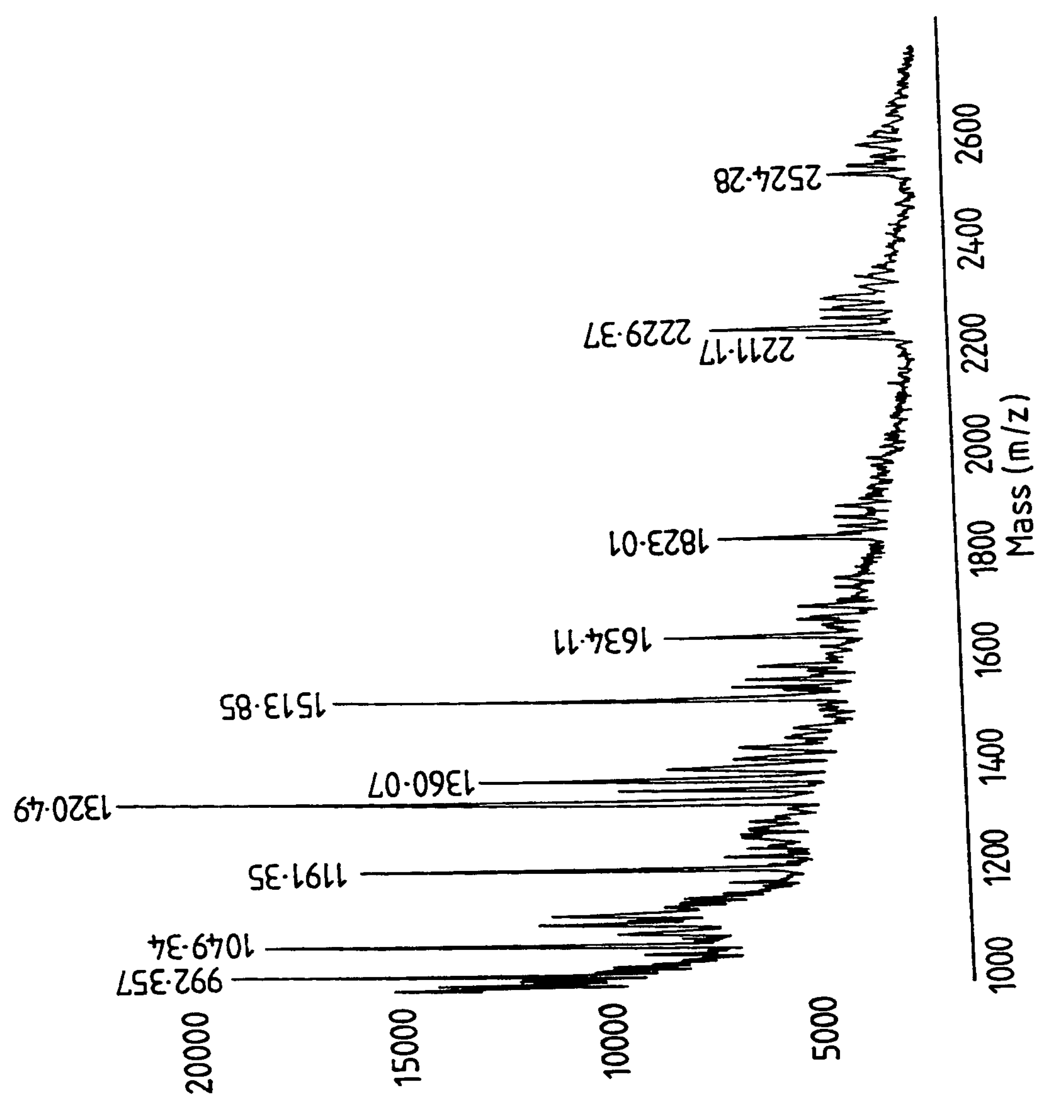
992·357
1049·34
1191·35
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1360·07
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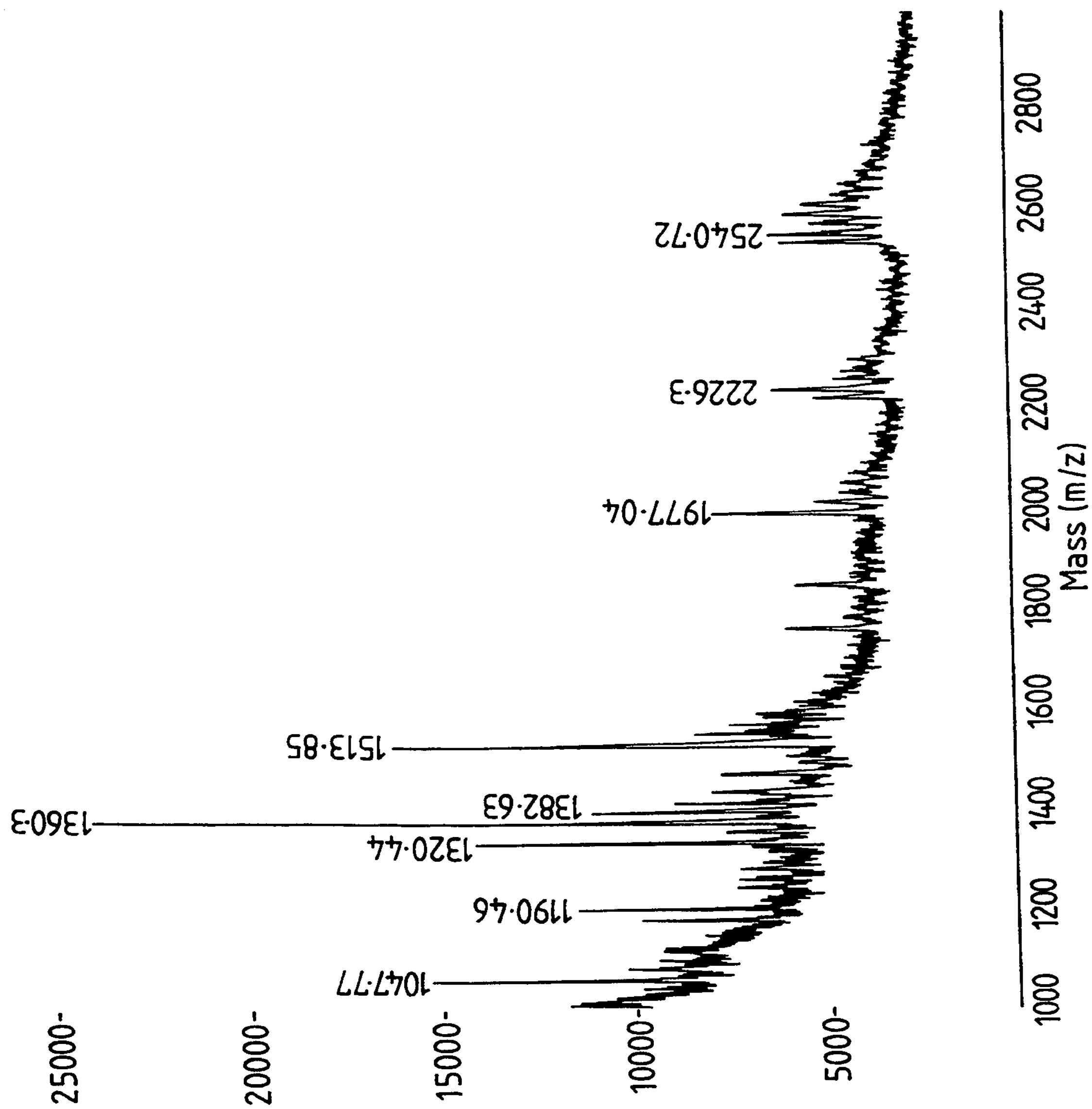
1634·11
1823·01
2211·17
2229·37
2524·28
20000
15000
10000
5000
1000
1200
1400
1600
1800
2000
2200
2400
2600
Mass (m/z)

FIG 2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1977·04
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25000-
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15000-
10000-
5000-
1000
1200
1400
1600
1800
2000
2200
2400
2600
2800
Mass (m/z)

FIG 3

CAC AAC GGA ATA GAC CCA AAA AGA UAA UUU CUA UCU
GUG UUG CCU UAU CUG GGU UUU UCU AUU AAA GAU AGA

GAU UUG CUU UAA AAC GUU UUU UUA GGA UCA CAA UGA
CUA AAC GAA AUU UUG CAA AAA AAU CCU AGU GUU ACU

UAU CUU UGC UGU AUU UGU AUA GUU CGA UGC UAA AUG
AUA GAA ACG ACA UAA ACA UAU CAA GCU ACG ATT TAC
*

CUC AUU GAA ACA AUC A
GAG TAA CTT TGT TAG T

FIG 4

Figure 5A

A

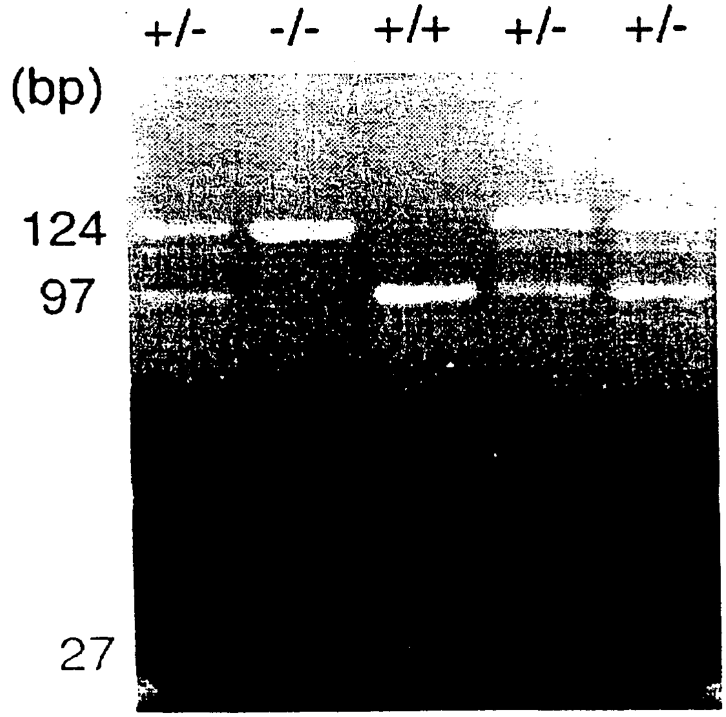
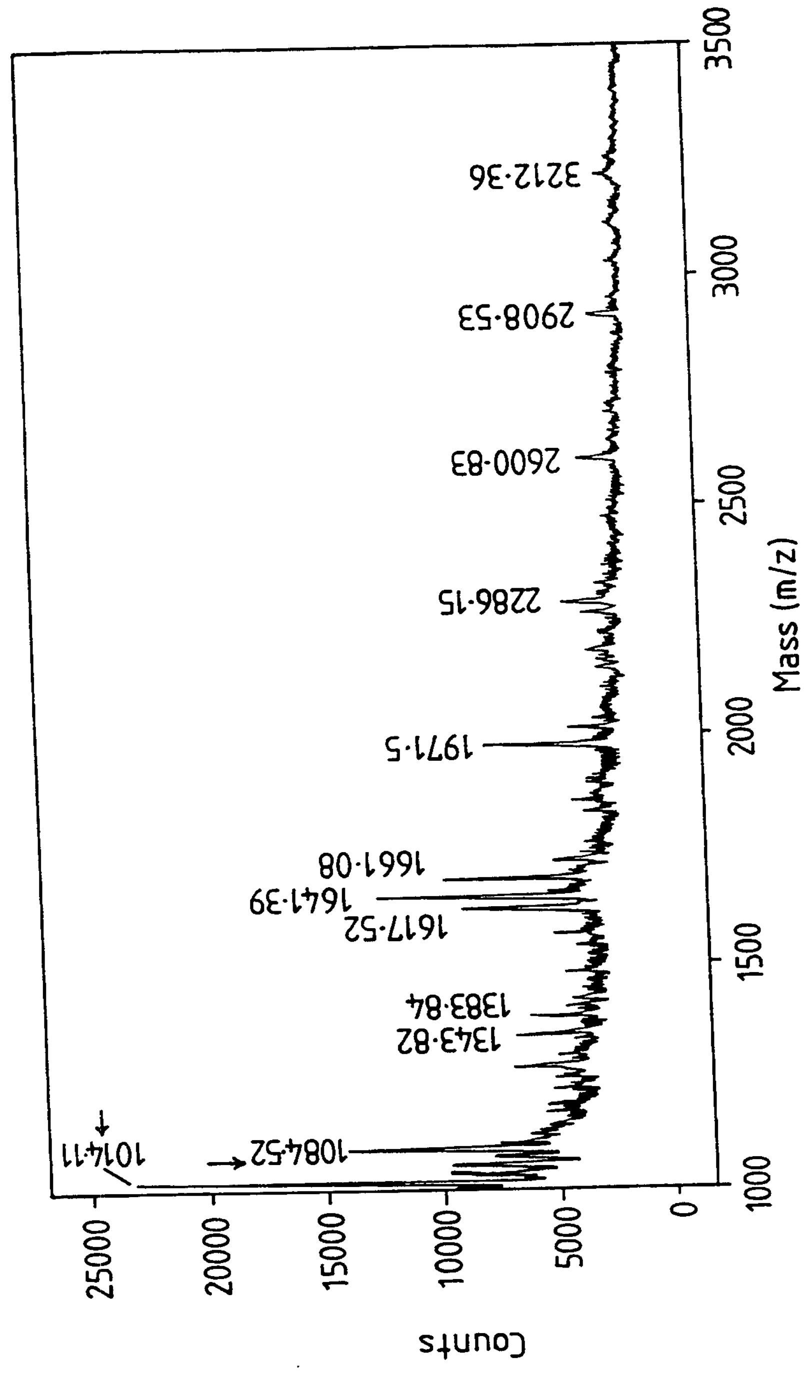


FIG 5B(i)a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Mass (m/z)
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1500
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2500
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3500
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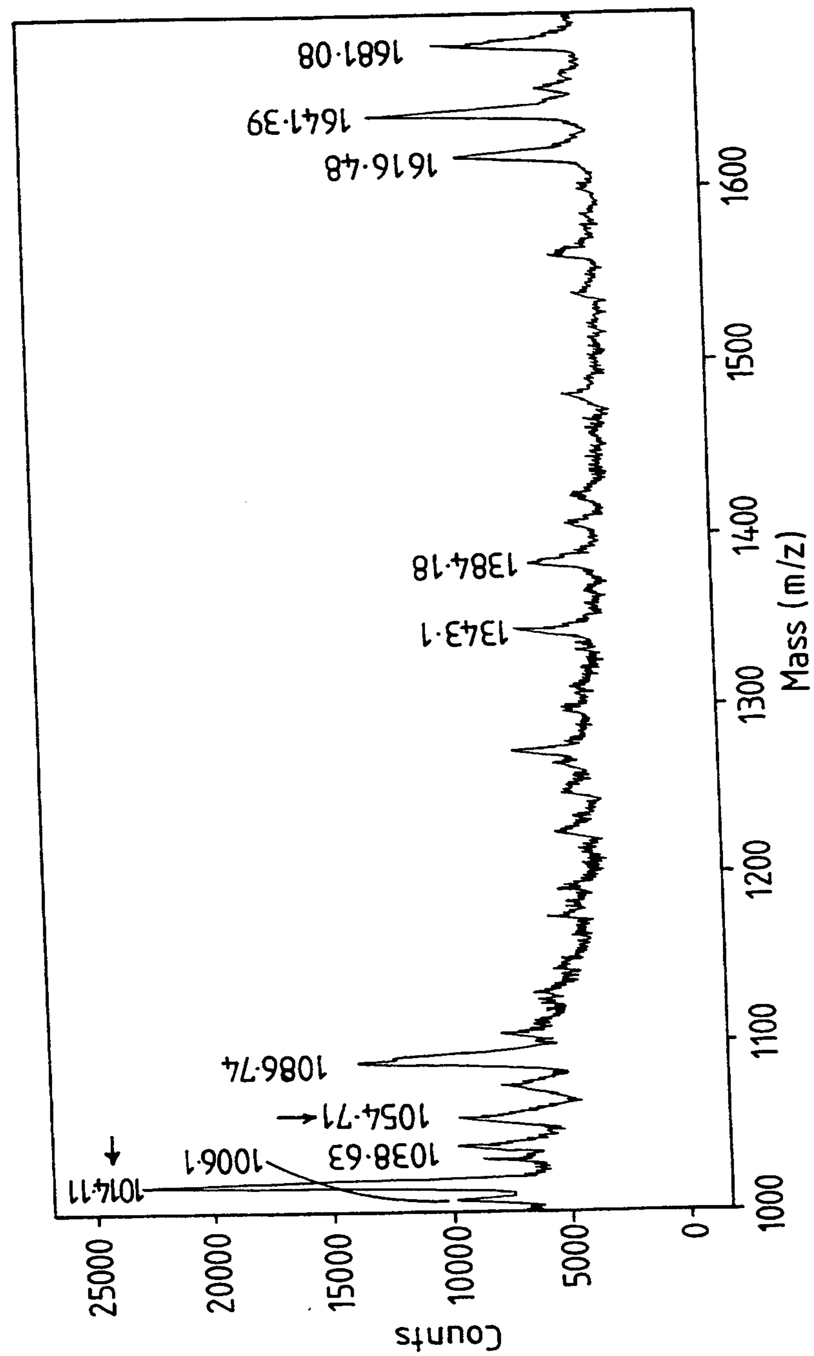
1084.52
1343.82
1383.84
1617.52
1641.39
1661.08
1971.5
2286.15
2600.83
2908.53
3212.36

FIG 5B(i)b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1006·1
1038·63
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1616·48
1641·39
1681·08
Counts
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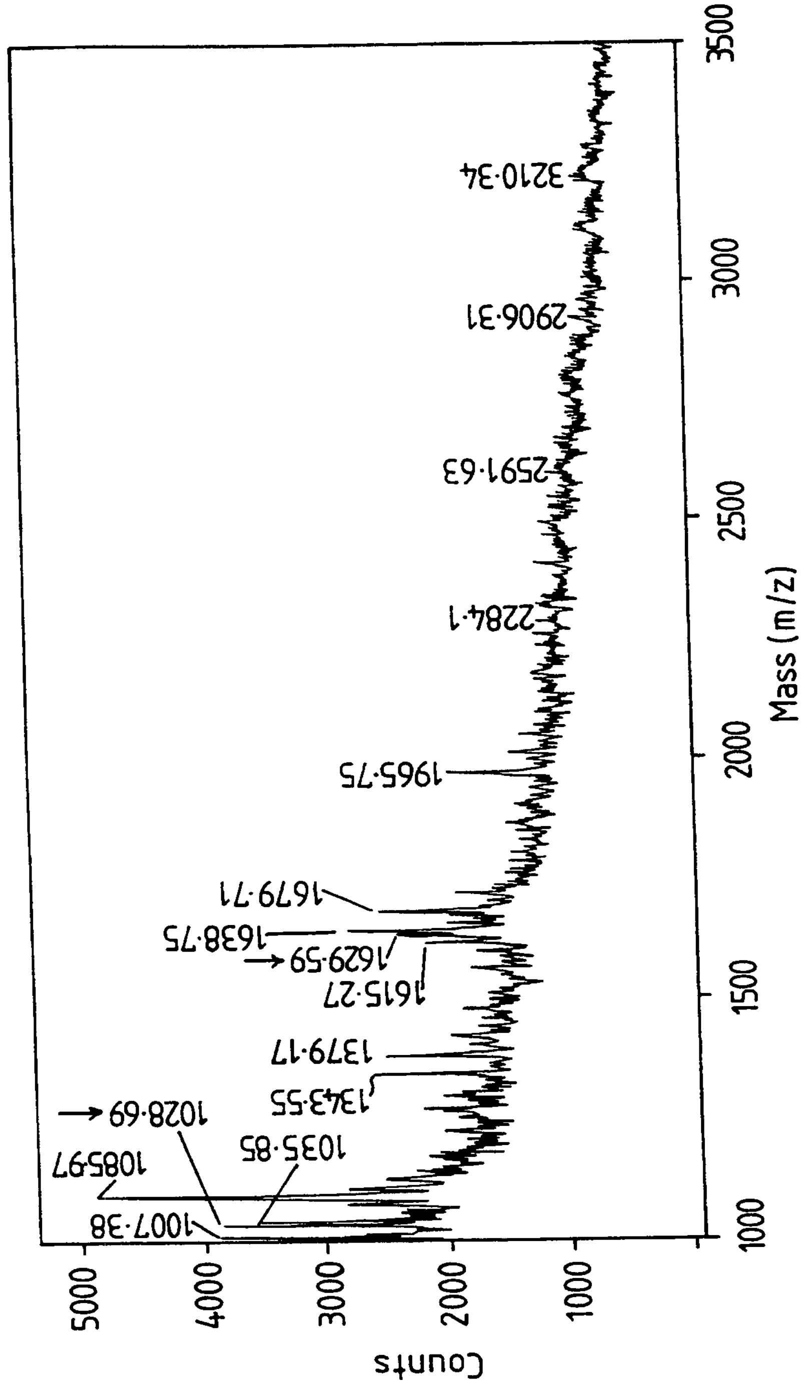
20000
15000
10000
5000
0
1000
1100
1200
1300
1400
1500
1600
Mass (m/z)

FIG 5B(ii)a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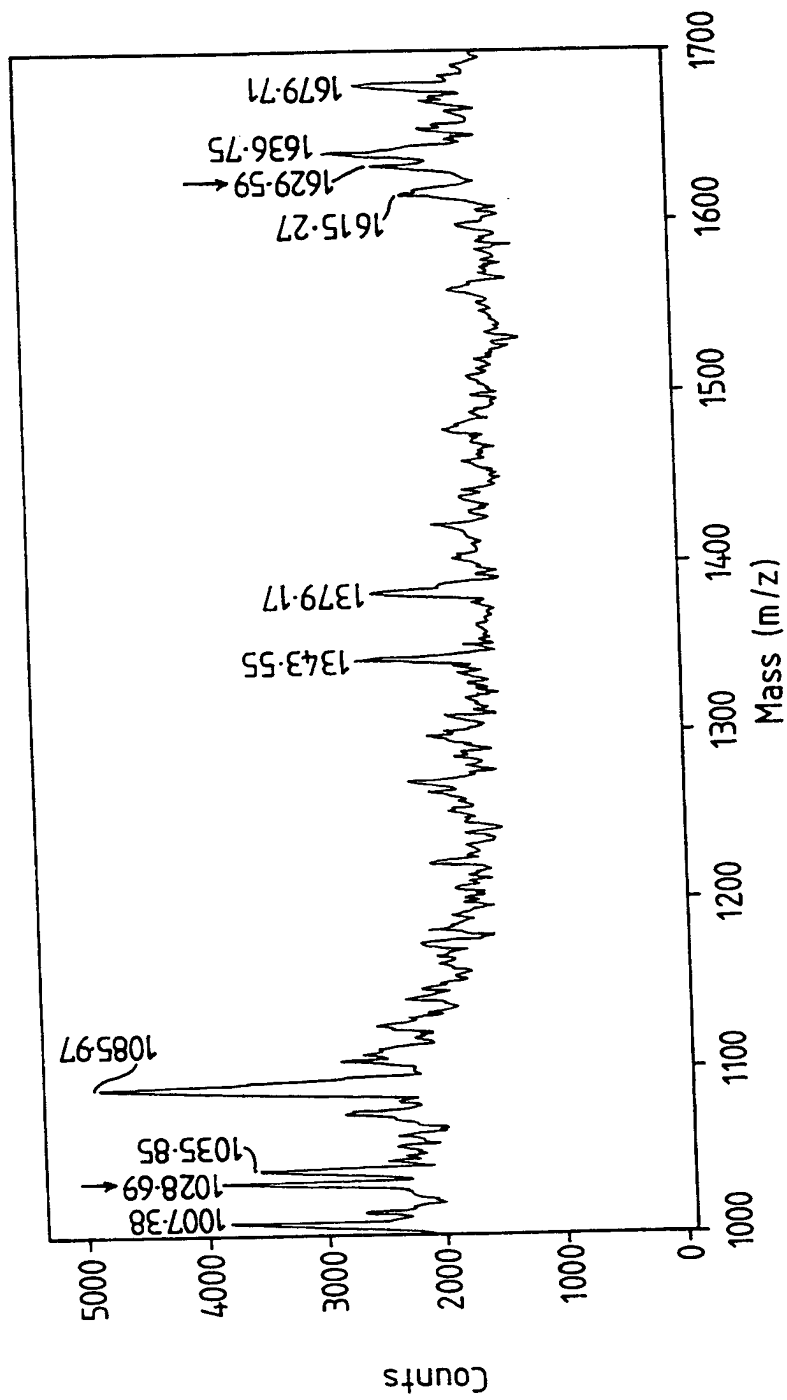
1085·97
1028·69
1035·85
1343·55
1379·17
1615·27
1629·59
1638·75
1679·71
1965·75
2284·1
2591·63
2906·31
3210·34
Counts
Mass (m/z)
5000
4000
3000
2000
1000
1500
2000
2500
3000
3500

FIG 5B(ii)b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1028·69
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1085·97
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1379·17
1615·27
1629·59
1636·75
1679·71
Mass (m/z)
Counts
1000
1100
1200
1300
1400
1500
1600
1700
0
1000
2000
3000
4000
5000

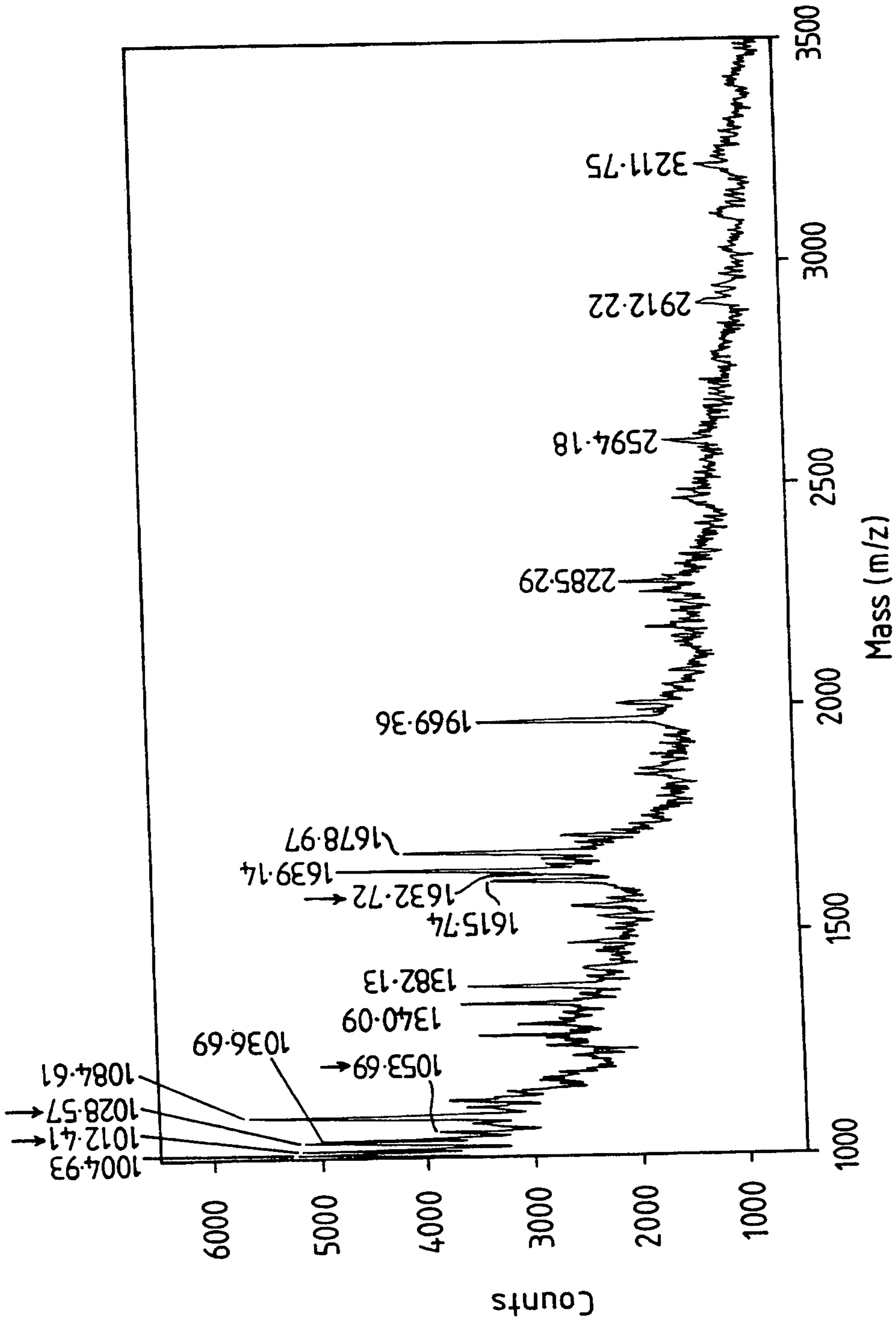
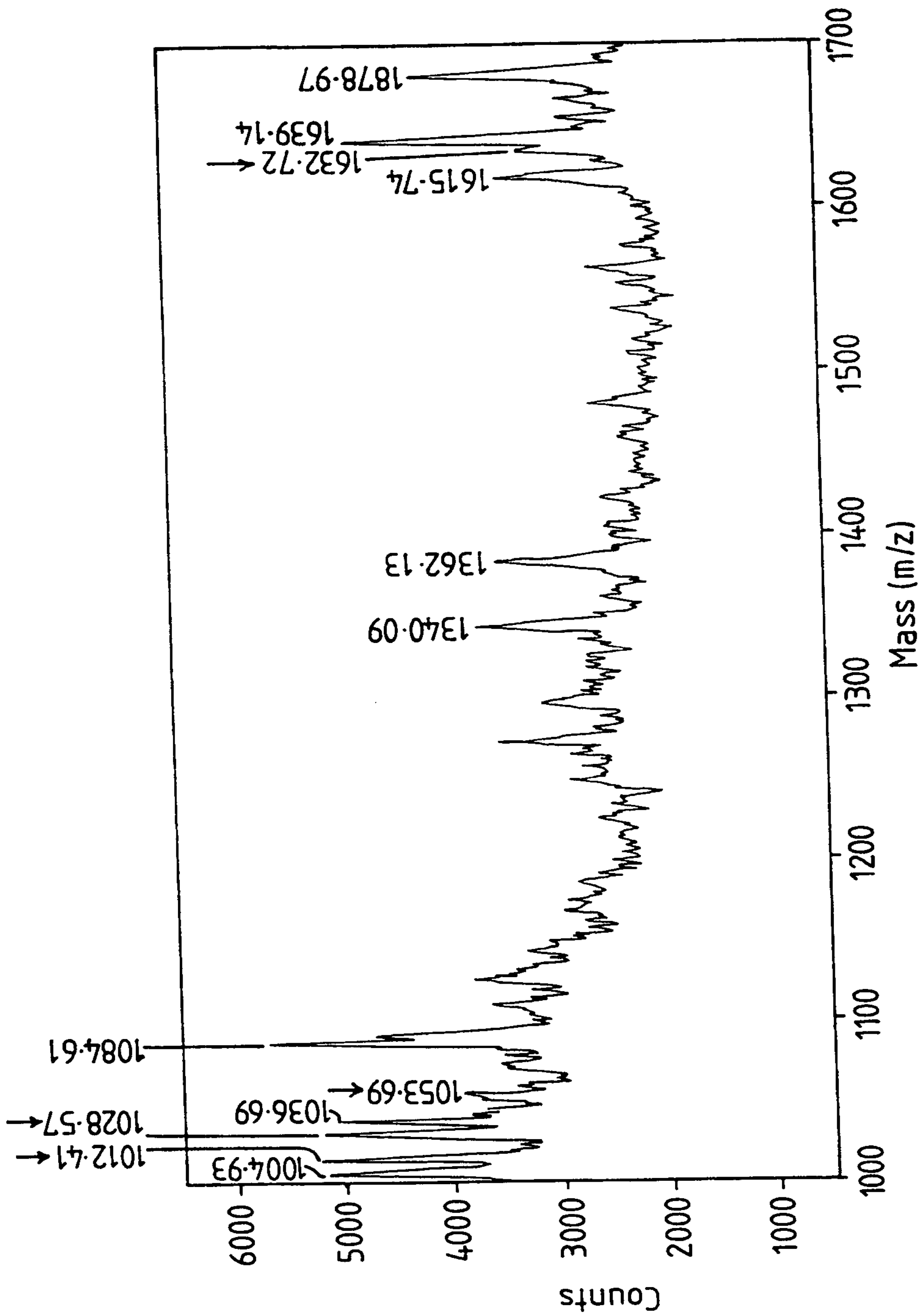

1004·93
1012·41
1028·57
1084·61
1036·69
1053·69
1340·09
1382·13
1615·74
1632·72
1639·14
1678·97
1969·36
2285·29
2594·18
2912·22
3211·75
Counts
Mass (m/z)
1000
1500
2000
2500
3000
3500
1000
2000
3000
4000
5000
6000

FIG 5B(iii)a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1012·41
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1362·13
1615·74
1632·72
1639·14
1878·97
Mass (m/z)
Counts
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1100
1200
1300
1400
1500
1600
1700
2000
3000
4000
5000
6000

FIG 5B(iii)b

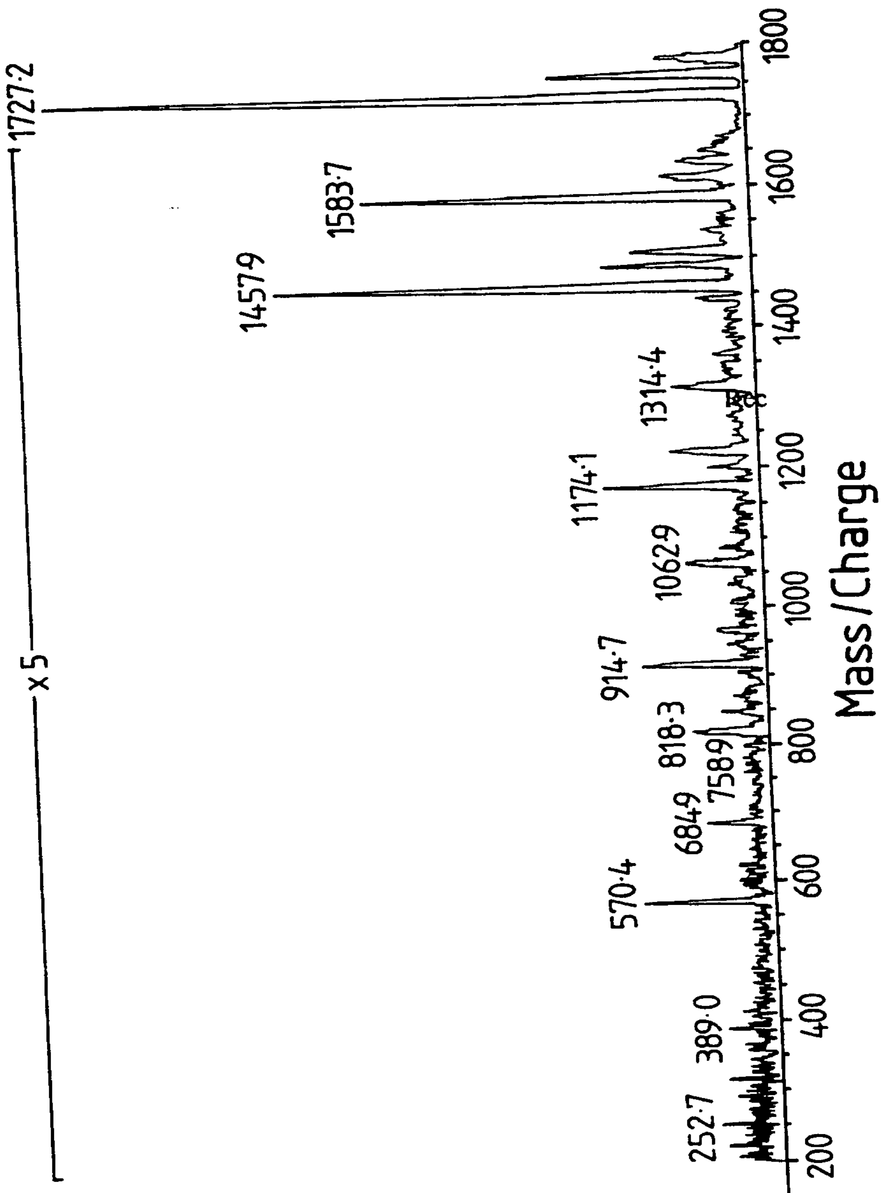
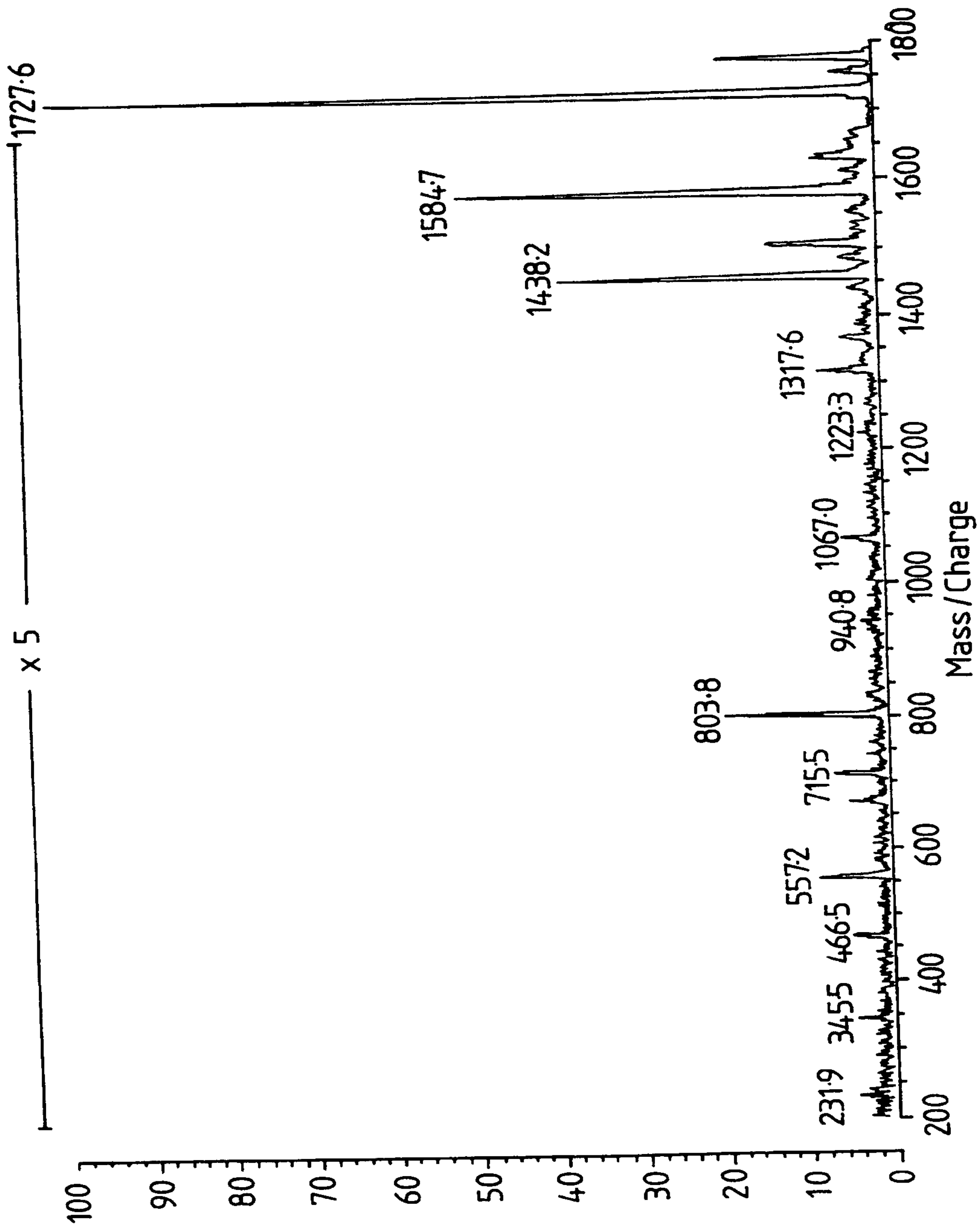

1727·2
1583·7
1457·9
1314·4
1174·1
1062·9
914·7
818·3
758·9
684·9
570·4
389·0
252·7
x 5
Mass/Charge
200
400
600
800
1000
1200
1400
1600
1800
0
10
20
30
40
50
60
70
80
90
100

FIG 6A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940·8
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715·5
557·2
466·5
345·5
231·9
x 5
Mass/Charge
200
400
600
800
1000
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1400
1600
1800
0
10
20
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100

FIG 6B

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00396

A.	**CLASSIFICATION OF SUBJECT MATTER**
Int Cl^{6}:	G01N 33/483, C12Q 1/527, 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC : G01N C12Q and keywords mass spectrom : and oligonucleotide or DNA or nucleic acid

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT (WPAT) and keywords as above
CHEMICAL ABSTRACTS (CA) and keywords as above

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/29431 A (SEQUENOM, INC) 26 September 1996 page 5 lines 13-21, page 6 lines 8-13, figs 7B, 9, 21A-C and claims 33 and 48	1-32
X	WO 96/36986 A (PERSEPTIVE BIOSYSTEMS, INC) 21 November 1996 whole document	1-32
X	WO 97/33000 A (GENETRACE SYSTEMS) 12 September 1997 page 10 line 1 to page 13 line 22, examples, 5, 8 and 11	1-32

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 August 1998	02.09.98

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00396

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98/12355, A (GENETRACE SYSTEMS) 26 March 1998 page 4 line 13 to page 6 line 32, page 9 lines 21 to page 10 line 7, examples 5 to 7	1-32
P,X	WO 98/20166 A (SEQUENOM, INC) 14 May 1998 abstract and claims	1-32

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 98/00396

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	96/29431	AU	53651/96	CA	2214359	EP	815261
		US	6505798				
WO	96/36986	EP	827628				
WO	97/33000	AU	20695/97				
WO	98/12355	AU	45916/97				
WO	98/20166	AU	51069/98				

END OF ANNEX